ISOLATION, IDENTIFICATION AND ANTIBIOTIC SENSITIVITY PATTERN OF PATHOGENIC ENTEROBACTERIA FROM TABLE EGG

Research Protocol

For Registration for Ph.D. Programme

Submitted by

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INTRODUCTION

The hen's egg is very rich in nutrients, making it one of the most valuable and most perfect foodstuffs. Eggs are considered to be an "excellent" source of choline and selenium and a "good" source of vitamin B12, phosphorus and riboflavin. The yolk contains vitamins A, D, E and K as well as folic acid, pantothenic acid and zinc [1]. Eggs can fully meet the requirements of organisms, including humans, for all the nutrients necessary for their development and life functions. At the same time many nutrient substances present in eggs create an excellent environment for the development of bacterial microflora, including pathogenic bacteria. The fact that eggs can be contaminated or infected horizontally (through the shell) or vertically (transovarially) makes them a potential source of pathogens participating in the etiology of food borne diseases in humans [2.]

Chemical composition of egg

Ingredient	Whole egg including Shell (%)	Whole egg excluding Shell (%)	Albumen (%)	Yolk (%)
Water	69.90	77.00	88.40	48.40
Protein	11.20	12.40	10.60	17.00
Fat	8.50	9.40	-	32.60
Carbohydrate	0.30	0.30	0.40	0.20
Ash	10.10	0.90	0.60	1.80

Freshly laid eggs are generally sterile, however they may constitute, if contaminated, a public health hazard, leading to losses from economic point of view through spoilage. Regarding the increasing consumption of egg and its products, it is necessary to investigate different factors implicated in egg contamination. The egg shell contamination may result from deposition of fecal material on the shell, ovarium or oviduct and gut flora, debris material, egg crates, packing and storage, clothes and hands of poultry workers, dust, the environment, weather conditions, transporting and marketing [3].

The microflora of the eggshell is dominated by Gram-positive bacteria, whereas Gram-negative bacteria are best equipped to overcome the antimicrobial defences of the egg content. In spite of the antibacterial factors, it can be infected with different bacteria such as *Salmonella* spp., *Listeria monocytogens*, *Campylobacter jejuni* and *Escherichia coli*. *Campylobacter* is the most common identified cause of food borne disease [4]. Much of the research on eggshell and egg content contamination focuses on Salmonella, since infection with *Salmonella enteritidis*, resulting from the consumption of contaminated eggs or egg products, is still a major health problem around the world. The significance of these diseases in humans can vary from mild symptoms to life threatening situation [5].

The absence of standard structures and drainage system in the market and other environmental factors such as temperature and humidity helps in bacterial penetration and increase the frequency of infection and spoilage because microbes grow within a range of 4°C and 60°C (optimum environment). Most retailers do not store eggs in refrigerators, they store eggs in an open room without any standard storage facilities, and thus the eggs are exposed to weather conditions, resulting in their contamination. Sanitation is also very poor as all sorts of animals enter and leave the market freely [6].

Pathogenicity is potential capacity of certain species of microbes to cause an infection. A microbe is either pathogenic or not, and is determined by its ability to produce toxins, ability to enter tissue and colonize and ability to spread from host to host [7].

The common contaminant organisms pathogenic to human beings include [6]:

- *Salmonella* spp.
- *Staphylococcus* spp.
- Gram positive *Bacillus*
- *Mucor*
- *Corynebacteria*
- *Aspergillus*
- *Escherichia coli*
- *Diplococci*

The Enterobacteria are heterogeneous group of gram-negative rods whose natural habitat is the intestinal tract of humans and animals. The family Enterobacteriaceae includes many genera (*Escherichia, Shigella, Salmonella, Enterobacter, Klebsiella, Serratia, Proteus*, and others) [2]. Some enteric organisms, eg, *Escherichia coli,* are part of the normal flora and incidentally cause disease, while others, the *Salmonella* and *Shigella* are regularly pathogenic for humans.

Salmonella spp. is the second most common cause of food poisoning after *Campylobacter* spp. It has been found in unpasteurised milk, eggs and raw egg products, meat and poultry. *Salmonella* spp. can be present inside of the completely normal-appearing eggs, and if the eggs are eaten raw or undercooked, the bacterium can cause illness, typhoid, enteric fever, septicaemia and gastroenteritis [8]. *Salmonella* is a common cause of bacterial food-borne gastroenteritis in many countries worldwide and in India it is known to be endemic. The overall annual incidence of food- borne Salmonellosis in India is nearly 6 per 1,000 inhabitants. Non-typhoid Salmonellosis originating from foods of animal origin is an emerging infection in India and is noted to be an increasing trend [9].

There are two main schools of thought regarding the entry of salmonellae into hatching eggs. The vertical transmission theory states that salmonellae come from an infected hen. The horizontal transmission theory states that salmonellae invade the egg through the shell after the egg is laid [4].

E. coli is one of the most common bacteria which cause diarrhoea especially in children. The elderly, infants, and those with impaired immune systems may have a more severe illness. In these patients, the infection may spread from the intestines to the blood stream, and then to other body sites and can cause death unless the person is treated promptly with antibiotics [5]. *E. coli* is known to contaminate the surface of egg while mechanical process can spread the bacteria through eggs and meat. Contamination with the pathogen while in the field, occur through improperly decomposed manure, contaminated water and poor hygienic practices of the farm workers. *E. coli* causes mastitis, urinary tract infection, meningitis, pneumonia and peritonitis [6].

It has been observed that the shell membrane had higher number of bacteria than yolk. In heavily contaminated environment, the eggs get infected through trans shell contamination and bacteria are translocated from outer to the inner surface of the shell and bacteria grow quickly in the shell membrane because of the availability of iron[10]. Thus, contaminated eggs, if consumed raw or semi-raw may be responsible for sporadic or epidemic diseases [8].

An important factor influencing quantitative bacterial contamination of eggs is the temperature at which they are stored, because the safety of eggs depends on the number of bacterial cells on the shells and in the content of the egg and on the rate at which they multiply within it. The risks of illness resulting from consumption of contaminated eggs depend not only on the number of bacterial cells in the contents or on the shells of the eggs, but also on the type of bacteria [11]. Among the most widespread food-borne infections directly connected with egg consumption are *Salmonella* infections.

Although the enteric bacteria isolated from eggs are not universally considered to be pathogenic but they may present a health risk for consumers using raw or undercooked eggs in their diet. WHO predicts that serious problems with opportunistic infections may appear in the 21st century. For this reason it is necessary to educate the public about good sanitary practices in handling eggs and preparing them for consumption [12].

Thus, the present study is undertaken to determine the bacterial contamination of the albumin, yolk and shell of hen’s egg depending on their source, storage duration and temperature. The study comprises a qualitative analysis of gram-negative microorganisms (Enterobacteria) of the family Enterobacteriaceae.

PART LITERATURE REVIEW

El-Prince [13] isolated some pathogenic bacteria such as *Staphylococcus aureus, E. coli, Salmonella* spp*, Shigella* spp. and other Enterobacteriaceae from the shells and contents of marketable chicken eggs.

Humphrey T. J. [14] demonstrated that the albumin is the major contamination site in egg, however, Bichler *et al.* [15] reported that the yolk is the most frequently contaminated egg fraction.

Bufano-Nancy [16] reported that sources of egg contamination are numerous as egg may be infected before it is laid, and also outside by the fecal matter, the lining of the nest, wash water if the eggs are washed, handling and perhaps by the material in which eggs are packed. Furthermore, according to the Centers for Disease Control and Prevention (CDC), eggs are responsible for an estimated 2,30,000 cases of food-borne illness each year.

Przybylska [17] reported that food poisoning and food-borne infection following consumption of eggs or dishes containing eggs are usually caused by *Salmonella*, as well as *Staphylococcus aureus*, *Escherichia coli* and other coli bacilli.

Antimicrobial resistance is an increasingly global problem, and emerging antimicrobial resistance has become a public health issue worldwide. A variety of foods and environmental sources harbor bacteria that are resistant to one or more antimicrobial drugs used in human or veterinary medicine and in food-animal production (Bager F. *et al.*)[18].

Pradhan *et al*. [19] suggested that contamination and penetration of salmonellae into hatching eggs may comprise an important link in the transmission of these bacteria to growing birds, processed carcasses, and eventually to the consumer.

Prevalence of *Salmonella* was recorded in 6.1% of egg shells and 1.8% of egg contents in South India (Suresh *et al.*) [20]. Friend and Fransom [21] found that Salmonella species can persist for 4 to 5 years within the hatchery.

Abdullah[4] demonstrated the degree of contamination of table egg with bacteria of genus *Staphylococcus, E. coli, Salmonella, Streptococcus* and *Clostridia* according to

source of eggs. The study indicated a relatively high degree of contamination of table egg with *Staphylococcus* bacteria and Enterobacteriaceae both in yolk and on egg shell. And also found that concentration of bacterial growth is higher in winter and lesser in summer.

Al-Bahry *et al*. [3] described the three different mechanisms in bacterial penetration of the egg: Adherence to eggshell and penetration through the eggshell pores, survival in albumin, and multiplication and growth in yolk.

Contamination of eggs with *Salmonella*, which is closely correlated with the spread of these bacteria among poultry is currently, one of the most important factors constituting a risk to consumer health (Stępień-Pyśniak D.) [2]. Other Gram-negative bacteria, such as *E. coli*, *Enterobacter* spp., *Citrobacter* spp., *Klebsiella* spp., *Alcaligenes* spp., *Aeromonas* spp, and *Pseudomonas* spp., have also been found in eggs with intact or damaged shells, where they play a role in spoilage and can enter the food chain via eggs, leading to food poisoning or infection in consumers. With the exception of *S. enteritidis*, there is currently little information about the distribution of Gram-negative bacteria in particular parts of eggs. The influence of storage conditions and of the source of the eggs on their qualitative bacterial contamination is also unknown.

The review of literature thus shows that pathogenicity is a microbial process, in which several microbial species cause infections. Although, many studies have been conducted on contamination and pathogenicity of hen's egg all over the world. As yet no work has been done on the egg contamination and pathogenicity in Jaipur city, also the temperature varies in the city during summer & winter seasons and cases of gastroenteritis in hospitals are also very frequent, so the present study is undertaken. However, my objective will be to isolate, identify and study the antibiotic sensitivity pattern of pathogenic Enterobacteria (*Salmonella* & *E. coli*) from table egg and also to find out the ways to reduce the frequency of food-borne diseases.

STATEMENT OF THE RESEARCH PROBLEM

ISOLATION, IDENTIFICATION AND ANTIBIOTIC SENSITIVITY PATTERN OF PATHOGENIC ENTEROBACTERIA FROM TABLE EGG.

Antibiotic sensitivity is the susceptibility of bacteria to antibiotics.

Pathogenicity is potential capacity of certain species of microbes to cause an infection.

Enterobacteria are heterogeneous group of gram-negative rods of Family Enterobacteriaceae, whose natural habitat is the intestinal tract of humans and animals.

Table egg means edible eggs derived as a product of poultry husbandry.

OBJECTIVES OF THE STUDY

1. To isolate the pathogenic Enterobacteria from different components (egg shell, albumin and yolk) of hen's egg.
2. To identify and characterize the isolated Enterobacteria.
3. To check the antibiotic sensitivity pattern of isolated Enterobacteria.
4. To find out the sensitive layers in hen's egg.
5. To find out appropriate procedure to reduce the contamination in hen's egg.

SCOPE OF THE STUDY

The main scope of this work is isolation, identification and characterization of Enterobacteria from different components of hen's egg.

Antibiotic sensitivity pattern of isolated Enterobacteria will be checked.

The present study will highlight the extent of prevalence of contamination in hen's egg in jaipur city.

SIGNIFICANCE OF THE STUDY

- The data obtained from the study will lead to comparative information regarding the duration & temperature of storage of eggs resulting in contamination and in suggesting effective measures for minimizing and preventing such microbial contamination.
- The data will be helpful in creating awareness and discouraging general public for the consumption of raw or half cooked eggs to reduce infection in consumers.

LIMITATIONS OF THE STUDY

- The egg surface and egg content may possess large number of microorganisms but the study will be restricted to two most common medically important pathogenic Enterobacteria (i.e. *Salmonella* & *E. coli*) because of time constraint.
- Other contaminants like toxic chemicals, pesticides, drugs etc will not be isolated during this study because of time constraint and quantum of work.
- The study will be restricted to Jaipur city only.

STUDY AREA

The study will be carried out in Jaipur city. The population of Jaipur city is about 3,073,350 with a growth rate of 5.3%. Jaipur has a hot semi-arid climate with temperature variations during summer and winter seasons. So, the present study is undertaken to determine the contamination of table egg by food-borne pathogens of Enterobacteria in Jaipur city.

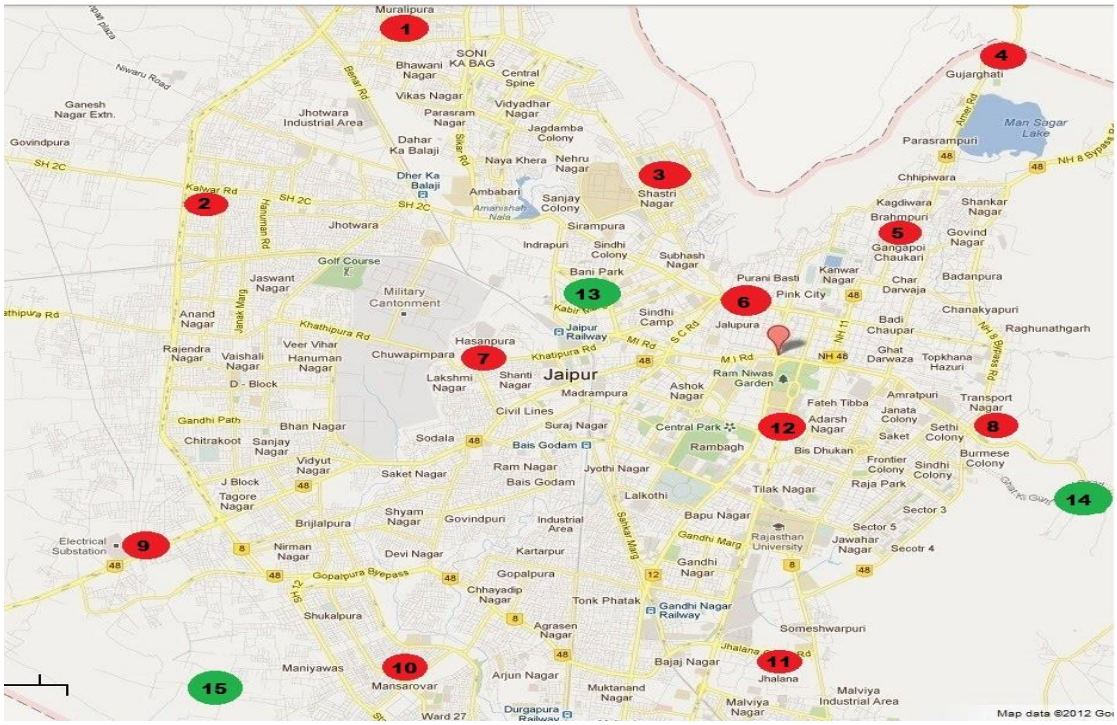


Fig. 1: Sample collection area map of Jaipur City (Red mark for area and green mark for Poultry farm)

Sample collection sites of Jaipur city are:

A. Areas

1. Murlipura
2. Kalwar Road
3. Shastri Nagar
4. Amber road
5. Bhrampuri
6. Chandpol
7. Hasanpura
8. Transport Nagar
9. Heerapura
10. Mansarovar
11. Jhalana
12. SMS Hospital

B. Poultry Farms

13. At Banipark
14. At Agra road
15. At Sanganer

METHODOLOGY

To carry out my research work the following methodology would be undertaken:

1. Sample Collection

Unfertilized eggs will be collected from total 15 different sites of Jaipur city (as given above and marked on the map) including malls or supermarkets, road side vendors, dairies and poultry farms.

From each sites, 10 eggs (both fresh and stored) will be randomly taken at a regular interval of two months for one year.

Besides the sample collection for experimental work, general information regarding the storage temperature and duration of the eggs will also be gathered from the sample collected sites.

The eggs will be collected in sterile containers and directed to the Central Laboratory at JECRC University, Jaipur for further studies where various micro-organisms will be isolated and identified. All the experimental work will be performed under standard laboratory conditions.

2. Sample Processing

The sterile plastic bags containing collected eggs will then be processed for culturing. Swab technique will be used to sample the shell surface of the intact eggs. Sterile cotton swabs dipped in sterile buffered peptone water (BPW) will be used to swab the entire surface area of the eggshell. The swabs will be directly inoculated into 10 ml BPW in screw-capped bottles. The same eggs from which shell sample is collected will be used for interior egg content sampling. In the culture of the egg contents (yolk & albumin), surface of each of the eggs will be first disinfected with 70% ethanol [6].

3. Isolation of Enterobacteria

Bacterial isolation will be performed by the conventional plating method. Qualitative determination of Gram-negative microorganisms, with particular consideration given to the family Enterobacteriaceae**,** will be carried out on selective and differential media (i.e. Salmonella-Shigella agar, MacConkey agar, EMB agar and Deoxycholate-citrate agar) at a temperature of 37^{o}C for 24-48 hours [22].

4. Identification of Enterobacteria[23]

The bacterial isolates will be identified according to the following parameters:-

A) Morphological identification

i) Culture characteristics (colony shape, size, color and odour).

ii) Gram's staining - to detect Gram-positive and Gram-negative microorganisms.

B) Biochemical characterization

• Catalase test.

• Haemolysis in blood agar plates.

• Oxidase test.

• IMViC test

• Urease test.

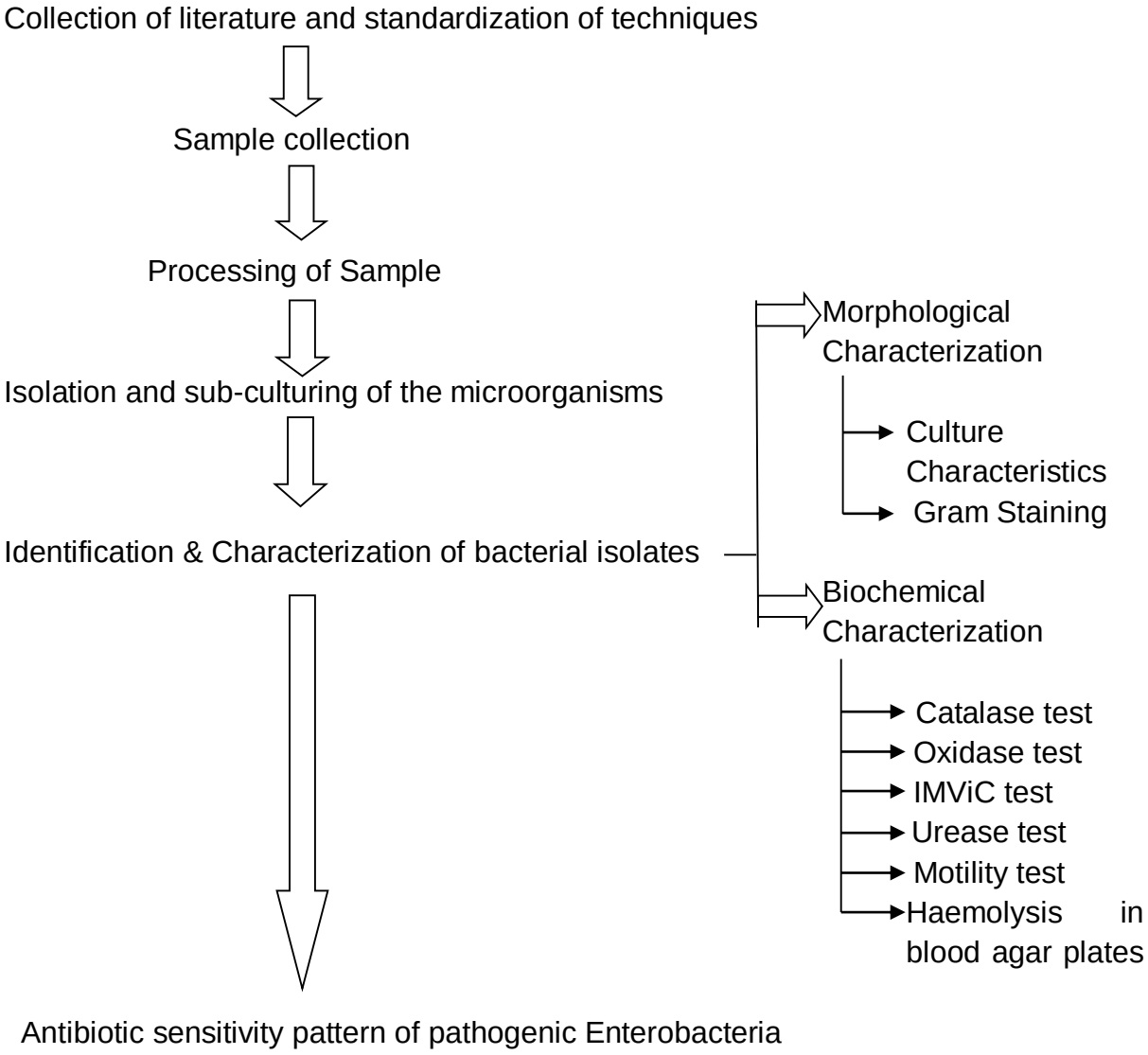
• Sugar fermentation of lactose, mannitol, glucose and sucrose.

• Motility test.

5. Antibiotic sensitivity test

A disc diffusion technique will be adopted [24] by using pure culture from the tested isolates and the test will be applied. The sensitivity of microorganisms to different antibiotic discs will be determined by measuring the diameter of inhibitory zones and compared with antibiotic susceptibility testing sheet to get the results (sensitive, intermediate or resistant). Inhibition zone will be expressed in diameter and measured after incubation at 37^0C for 24 hrs.

OUTLAY


Collection of literature and standardization of techniques
Sample collection
Processing of Sample
Isolation and sub-culturing of the microorganisms
Identification & Characterization of bacterial isolates
Morphological Characterization
Culture Characteristics
Gram Staining
Biochemical Characterization
Catalase test
Oxidase test
IMViC test
Urease test
Motility test
Haemolysis in blood agar plates
Antibiotic sensitivity pattern of pathogenic Enterobacteria

MONTH WISE PLAN OF WORK

Activity	Months
Review of Literature throughout the study	01 - 24months
Standardization of techniques	02 - 03 months
Sample collection and Culture Preparation	02 - 14 months
Isolation and sub-culturing of the microorganisms	02 - 14 months
Biochemical and morphological characterization of isolated Enterobacteria	02 - 14 months
Analysis of pathogenic potential of isolated Enterobacteria	14 - 16 months
Compilation, analysis and interpretation of Data	16 - 18 months
Thesis writing and Submission of Report	19 - 24months

Total time duration (expected) = 24 months

WORK PLAN (GRAPHICAL REPRESENTATION)

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Collection of the Review of Literature throughout the study																								
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